Arabidopsis thaliana bZIP44: a transcription factor affecting seed germination and expression of the mannanase-encoding gene *AtMAN7*

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SUMMARY

Endo-β-mannanases (MAN; EC. 3.2.1.78) catalyze the cleavage of β1→4 bonds in mannan polymers and have been associated with the process of weakening the tissues surrounding the embryo during seed germination. In germinating *Arabidopsis thaliana* seeds, the most highly expressed *MAN* gene is *AtMAN7* and its transcripts are restricted to the micropylar endosperm and to the radicle tip just before radicle emergence. Mutants with a T-DNA insertion in *AtMAN7* have a slower germination than the wild type. To gain insight into the transcriptional regulation of the *AtMAN7* gene, a bioinformatic search for conserved non-coding *cis*-elements (phylogenetic shadowing) within the Brassicaceae *MAN7* gene promoters has been done, and these conserved motifs have been used as bait to look for their interacting transcription factors (TFs), using as a prey an arrayed yeast library from *A. thaliana*. The basic-leucine zipper TF AtbZIP44, but not the closely related AtbZIP11, has thus been identified and its transcriptional activation upon *AtMAN7* has been validated at the molecular level. In the knock-out lines of *AtbZIP44*, not only is the expression of the *AtMAN7* gene drastically reduced, but these mutants have a significantly slower germination than the wild type, being affected in the two phases of the germination process, both in the rupture of the seed coat and in the breakage of the micropylar endosperm cell walls. In the over-expression lines the opposite phenotype is observed.

Keywords: transcription regulation, *AtbZIP44*, *AtbZIP11*, *AtMAN7*, seeds, germination, endosperm rupture, seed coat rupture, *Arabidopsis thaliana*.

INTRODUCTION

In mature angiosperm seeds, the diploid embryo and the triploid endosperm are surrounded and protected by the maternal tissue of the seed coat (testa). In *Arabidopsis thaliana* and other Brassicaceae species, seeds contain an endosperm of one to three cell layers and germination proceeds in two differentially regulated steps, where testa rupture is followed by endosperm breakage that allows the elongating radicle to protrude (germination *sensu stricto*). Thus, endosperm rupture (ER) and seed coat rupture (SCR) are two distinct limiting factors for germination to occur (Müller *et al.*, 2006; Bentsink and Koornneef, 2008; Holdsworth *et al.*, 2008; Piskurewicz *et al.*, 2009; Iglesias-Fernández and Matilla, 2010). While SCR is not affected by abscisic acid (ABA), this hormone specifically inhibits ER; gibberellins (GA), which are ABA antagonists, affect both SCR and ER. Although the upstream GA-signalling components have been identified recently, the *cis*-elements and *trans*-acting factors that regulate downstream GA-responsive genes need further investigation (Sun and Gubler, 2004). Other hormones such as ethylene and brassinosteroids also influence germination, but the GA/ABA ratio is the most important factor for the integration of the environmental signals for the occurrence of germination (Kucera *et al.*, 2005; Matilla and Matilla-Vázquez, 2008; Linkies *et al.*, 2009). While the driving force for radicle elongation has been described as the main factor influencing SCR, cell wall (CW)-modifying enzymes, specifically those localized at the micropylar endosperm (ME), are considered main players in ER. These enzymes include cellulases, endo-β(1→4)-mannanases (MANs), chitinases, peroxidases, expansins,

xyloglucan endotransglycosylase/hydrolases (XTH), etc. (Petruzzelli *et al.*, 2003; Nonogaki *et al.*, 2007; Iglesias-Fernández *et al.*, 2011a; Martínez-Andújar *et al.*, 2012).

The enzymatic activity of MAN and expression of several members of the *MAN* gene family have been characterized in many plant species because of their important role in the germination of mannan-rich seeds (Rodríguez-Gacio *et al.*, 2012). In *Arabidopsis thaliana*, four *MAN* genes (*AtMAN2*, *AtMAN5*, *AtMAN6* and *AtMAN7*) are expressed in germinating seeds, the *AtMAN7* transcripts being the most abundantly accumulated through this process. The *AtMAN7* *mRNA*s are restricted to the ME and to the radicle tip, disappearing as soon as the radicle emerges. Moreover, knock-out (KO) mutants in *AtMAN7* have a significantly slower germination than wild-type seeds, suggesting a putative role for this gene in this biological process (Iglesias-Fernández *et al.*, 2011a). Since *AtMAN* genes have predicted signal peptides, and therefore their corresponding proteins could be secreted, it is reasonable to suggest an interaction between the radicle tip of the embryo and the ME MAN enzymes in the dismantling of the mannan-rich seed CWs in order to facilitate radicle protrusion (Iglesias-Fernández *et al.*, 2011b).

Transcriptional regulation of gene expression is a key regulatory process in any living organism that is driven by short DNA sequences (*cis*-elements) situated in gene promoters and transcription factors (TFs), proteins that recognize and bind to these *cis*-elements. Identification of the *cis*-regulatory code and the TFs that interact with them (*trans*-regulators) is the main challenge in unveiling any combinatorial transcriptional regulatory network (Singh, 1998). Non-coding sequences in gene promoters diverge rapidly during evolution, except for those that are functionally important. *In silico* comparison of the promoter sequences of orthologous genes from several related species can be used to find these conserved non-coding elements. This 'phylogenetic shadowing' process has been described in *Saccharomyces* (Cliften *et al.*, 2001), in primates (Boffelli *et al.*, 2003) and in *A. thaliana* and related Brassicaceae (Hong *et al.*, 2003). Coupling these *in silico* analyses with screenings of an arrayed library of *A. thaliana* TFs in yeast (one-hybrid assays; Y1H) has unveiled novel *cis–trans* interactions (Castrillo *et al.*, 2011, and references therein).

To gain insight into the transcriptional regulation of the *AtMAN7* gene during seed germination, a bioinformatic search for its orthologous genes and corresponding promoters within the Brassicaceae family has been done. In *A. thaliana*, *Arabidopsis lyrata*, *Capsella rubella*, *Brassica rapa*, *Thellungiella halophila* and *Arabis alpina*, a highly conserved non-coding *cis*-element within the promoter regions of the *MAN7* genes has been identified by phylogenetic shadowing and shown to be functionally relevant *in planta*. This *cis*-element has been used as a bait to look for its interacting TFs (Castrillo *et al.*, 2011). The basic-leucine zipper AtbZIP44, but not its closely related AtbZIP11, has been thus identified and its regulatory function upon *AtMAN7* validated by RT-qPCR analyses, mRNA fluorescence *in situ* hybridization (FISH) experiments and by the germination kinetics of both over-expression (oex) lines and T-DNA insertion mutants in the *AtbZIP44* gene. The different oex-*bZIP44* lines show faster germination than the wild type; moreover, KO lines in the *AtbZIP44* gene analyzed not only present drastically reduced *AtMAN7* transcript content but display a slower germination than the wild type in the two parameters analyzed: SCR and ER.

RESULTS

The *AtMAN7* gene promoter contains evolutionarily conserved *cis*-elements and responds to GA but not to ABA

The *A. thaliana MAN* gene family (*AtMAN*), that is represented by seven members (Iglesias-Fernández *et al.*, 2011a), has been used to search for orthologous genes in related Brassicaceae species (Table S1 in Supporting Information). The corresponding deduced protein sequences have been used to construct an unrooted phylogenetic tree using the neighbor-joining algorithm (Figure S1). The 37 sequences compared are grouped into four major clusters of orthologous genes (MCOGs; A, B, C and D), supported by bootstrap values higher than 70% and by the occurrence of common protein motives shared by members of the same MCOG (Table S2). The MEME analysis indicates that all the MAN proteins annotated share motifs 1, 5, 6, 7 and 8 where amino acids described as critical for enzyme activity are present (Yuan *et al.*, 2007) and that motif 11 spans a putative signal peptide (Figure S1, Tables S1 and S2). The physiological significance of this signal peptide has been validated by the subcellular localization of AtMAN7. For this purpose the *AtMAN7* open reading frame (ORF) has been translationally fused to that of green fluorescence protein (GFP) under the control of the constitutive 35s promoter (*P35s::AtMAN7-GFP*) and this construct used in transient expression experiments in bombarded onion epidermal cells. As shown in Figure S2, the green fluorescent signal appears in the peripheral regions of the cell (cell wall and plasma membrane; arrows), as expected from its signal peptide.

The promoter region of the *AtMAN7* gene (*At5 g66460*) has been searched for conserved *cis*-elements, putatively involved in its transcriptional control, through the promoters of its gene orthologs (Figure 1, Table S3). The length of the DNA sequence compared was 844 bp, considering the intergenic distance between the ATG translation initiation codon of the *AtMAN7* (*At5 g66460*) and its preceding gene *At5 g66470* in the *A. thaliana* genome (Figure 1a). The pair-wise alignment among the six orthologous gene

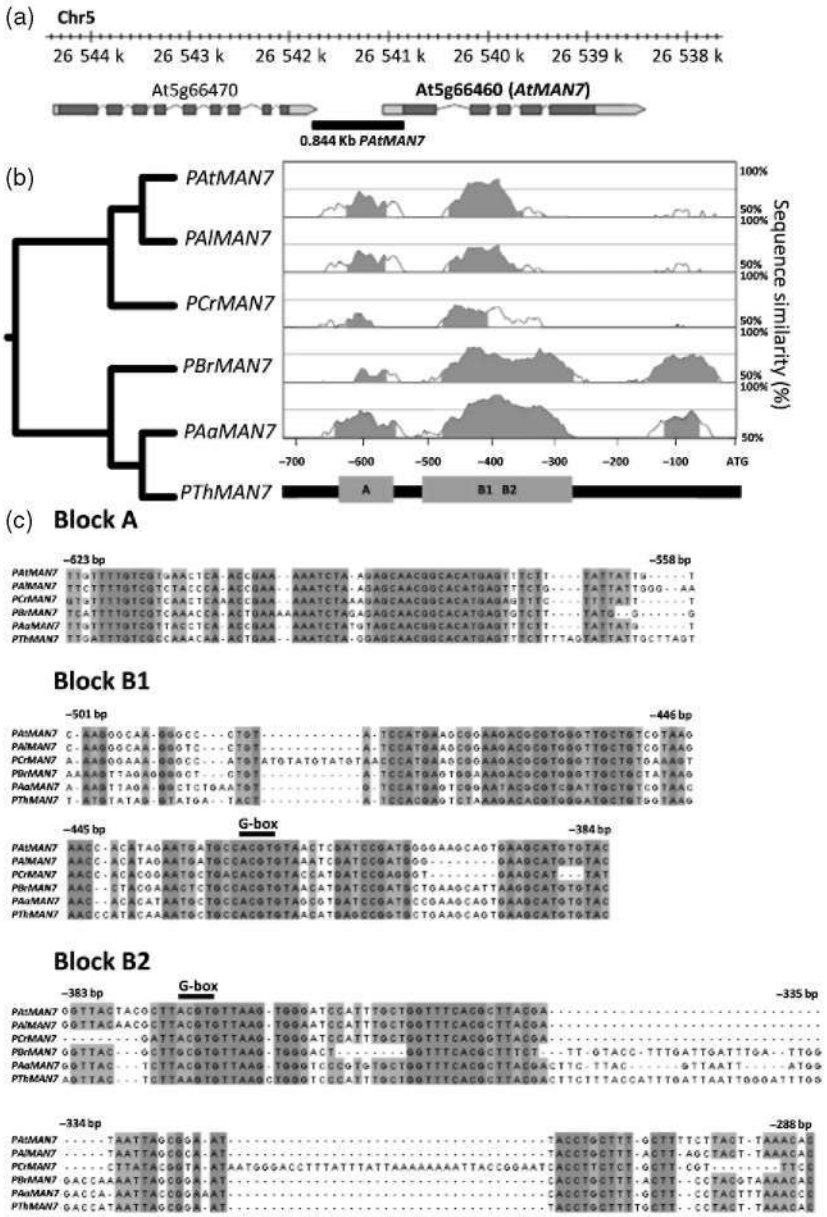

Figure 1. Identification of conserved *cis*-elements in *MAN7* promoters.
(a) Genome browser view of the *MAN7* locus on chromosome 5 in the *Arabidopsis thaliana* accession Col. Exons of *MAN7* and the flanking gene *At5 g66470* are represented as gray boxes and untranslated regions as lines. Arrows indicate the direction of transcription. The promoter sequence used for pairwise alignment is represented by a black box.
(b) Pairwise alignment of *MAN7* promoter sequences from different Brassicaceae species using mVISTA. Graphical output shows base pair identity in a sliding window of 70 bp of 70%. Grey areas show conserved blocks (A, B1 and B2).
(c) Sequence alignment of the block A, B1 and B2 conserved residues in the *MAN7* promoter. G-boxes are indicated.

promoters compared reveals two highly conserved *cis*-elements that have been named block A (65 bp) and block B (213 bp; Figure 1b). Block A, spans from −623 to −558 bp upstream of the ATG and block B is situated between −501 and −288 bp and their nucleotide comparison is shown in Figure 1(c).

In order to explore the functional relevance of these conserved motifs, a set of deletion constructs of the *AtMAN7* promoter (*PMAN7*) have been generated, fused to the β-glucuronidase (GUS) reporter gene (*uidA*). These constructs are *PMAN7-1::uidA* (−844 bp) containing the whole promoter; *PMAN7-2::uidA* (−509 bp) that lacks the conserved motif A and *PMAN7-3::uidA* (−280 bp) that is deprived of blocks A and B (Figure 2a). Homozygous transgenic *A. thaliana* plants have been generated with these constructs and histochemical GUS expression evaluated (Figure 2b). The transgenic lines with the *PMAN7-1::uidA* construct shows that *uidA* is present in leaves (hydathodes) and flowers (anther filaments, stigma, vascular elements of sepals, etc.), it is strongly expressed in the vascular elements of the roots, both in seedlings and in adult plants, and at the base and apical parts of siliques and at the replum (Figures 2b and S3). During germination, GUS activity is restricted to the ME at 24 h of germination. This *uidA* expression is conserved in transgenic lines where block A has been eliminated (*PMAN7-2::uidA;* −509 bp) and completely disappears in both germinating seeds and in adult plants where the conserved blocks A and B have been

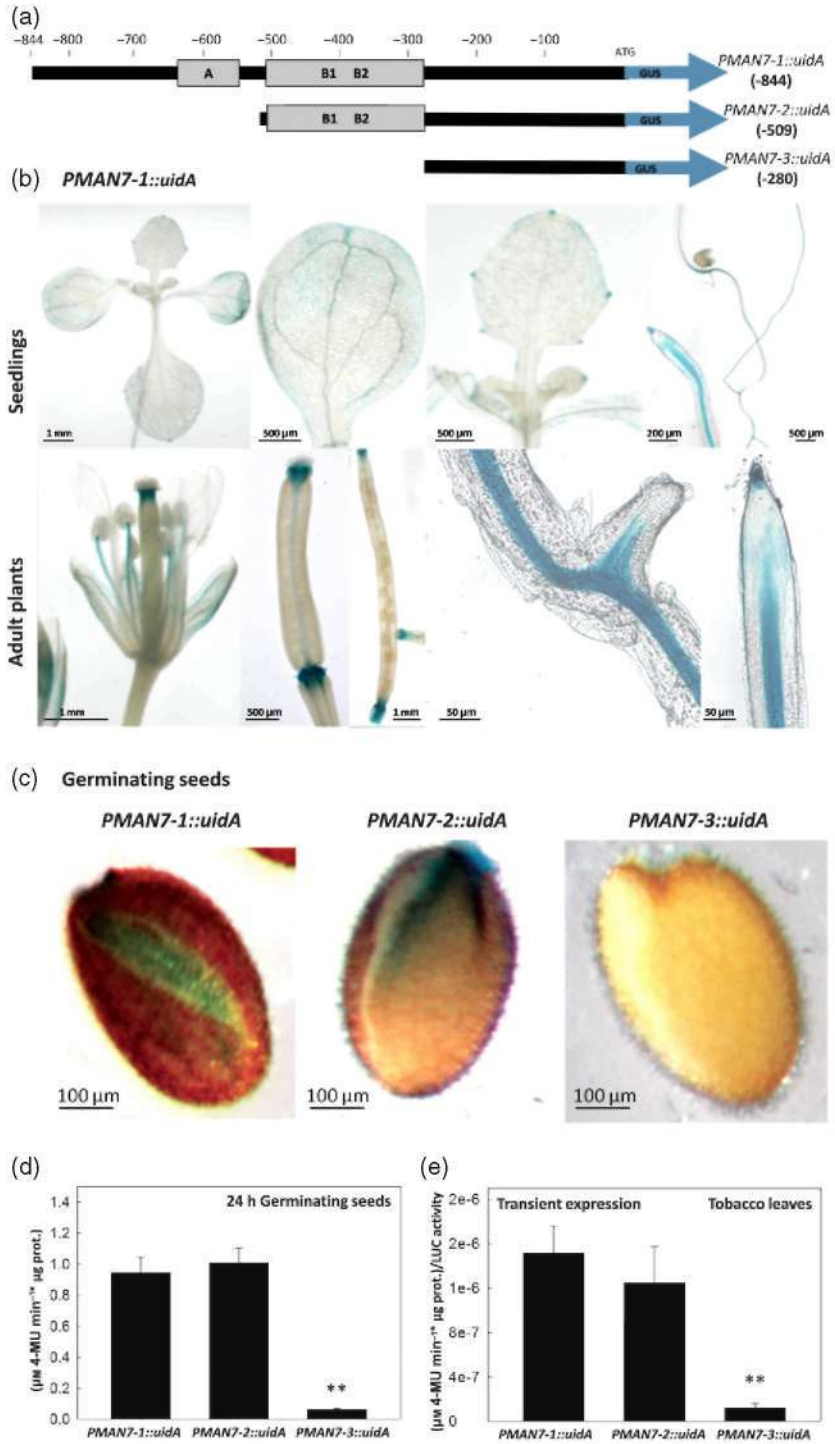

Figure 2. Expression of GUS (*uidA*) driven by different fragments of the *AtMAN7* gene promoter.
(a) Schematic representation of the three reporter constructs used in stable and transient expression assays.
(b) Histochemical localization of GUS in *PMAN7-1::uidA* transgenic lines in seedlings and adult plants.
(c) Histochemical localization of GUS in 24-h germinating transgenic seeds with the three constructs described in (a).
(d) GUS quantification in 24-h germinating seeds. Data are means ± standard error (SE) of at least three replicates in two independent transgenic lines for each construction. Asterisks indicate significant difference (*P*-value < 0.01).
(e) Transient expression assays in *Nicotiana benthamiana* leaves with the three constructs described in (a). Data are means of ± SE of at least six replicates for each construction. Asterisks indicate significant difference (*P*-value < 0.01).

deleted (*PMAN7-3::uidA*; −280 bp; Figures 2c and S3). In Figure 2d, the *uidA* expression in 24-h germinating seeds from the homozygous lines for each one of these three constructs has been quantified. These results have been further corroborated by transient expression assays by agro-infiltration in *Nicotiana benthamiana* leaves using the same three constructs of the *AtMAN7* promoter (Figure 2e). Taken together, these results indicate that block B contains important regulatory *cis*-elements for the transcriptional regulation of the *AtMAN7* gene.

The same stable *A. thaliana* transgenic lines described in Figure 2a have been used to quantify GUS upon seed germination under different hormonal treatments (50 µM GA_{4+7}, 1 µM ABA and water as a control; Figure 3a–i). In

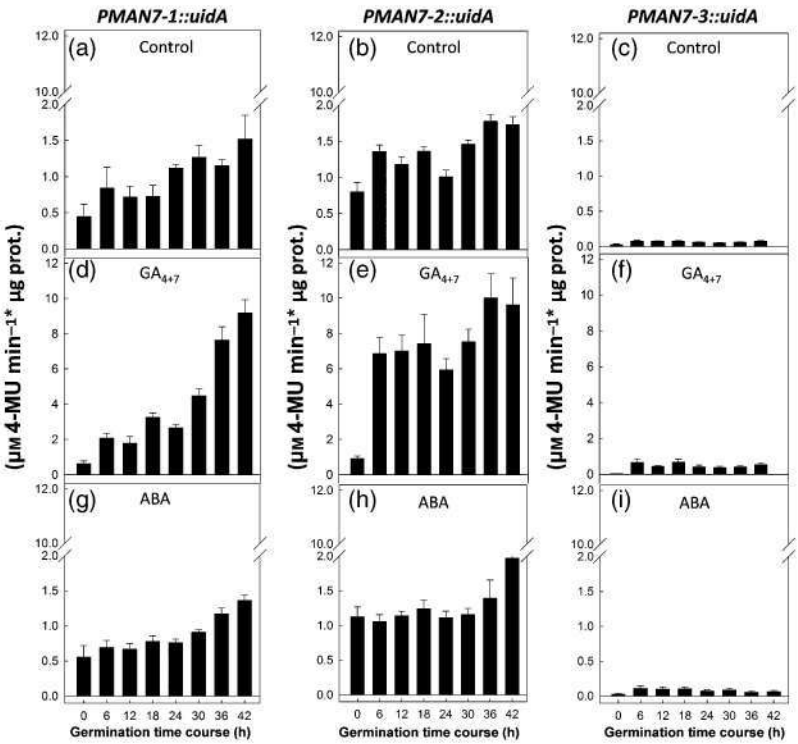

Figure 3. The *AtMAN7* gene promoter responds to gibberellin GA_{4+7}. The GUS activity in *Arabidopsis thaliana* transgenic seeds at different time points of germination in sterile water (control; a–c), 50 μM GA_{4+7} (d–f) and 1 μM ABA (g–i). Data are means ± standard error (SE) of at least three replicates in two independent transgenic lines for each construction.

PMAN7-1::uidA germinating seeds, the addition of GA_{4+7} to the imbibition medium produces a significant enhancement of GUS activity, compared to the controls at all time points analyzed (Figure 3a,d); this increment in GUS activity is particularly remarkable at 18-h (three-fold higher in the GA_{4+7}-treated seeds than in the water controls) and at 36 h, where a peak of activity of one order of magnitude higher than controls is reached. Significantly, in *PMAN7-2::uidA* (−509 bp) germinating seeds, GA_{4+7} provokes an earlier (6 h) enhancement of GUS activity (one order of magnitude higher than seeds with the *PMAN7-1::uidA* construct; Figure 3b,e), which suggest that block A contains a negative *cis*-motif of the *AtMAN7* gene GA response. Exogenous addition of ABA (1 μM) to the imbibition medium does not significantly modify GUS activity in any of the transgenic germinating seeds analyzed (Figure 3g,h). The *PMAN7-3::uidA* germinating seeds do not support any GUS activity (Figure 3c,f,i).

AtbZIP44, but not its paralog AtbZIP11, binds specifically to the G-box element within conserved block B1 in yeast-one hybrid assays

The conserved *cis*-element identified *in silico* as block B has been split into two blocks, B1 (118 bp) and B2 (96 bp), to be used as baits in the screening of the yeast TF library. This library contains a collection of about 1200 *A. thaliana* TF ORFs, arrayed in a 96-well format (RR library; REGIA + REGULATORS library) and a convenient mating system has been developed, based on the yeast-one hybrid (Y1H) screening procedure. An episomal plasmid (*pTUY1H*) has been used to clone the conserved B1 and B2 elements to be used as baits for the screening of this RR library (Castrillo *et al.*, 2011).

Following this procedure, the B2 element has not been recognized by any TF in our library (data not shown) and the basic-leucine zipper (bZIP) AtbZIP44 (*At1 g75390*) TF has been identified using the conserved B1 element as bait. This result has been further corroborated when using a smaller segment of 46 bp (spanning from −445 to −397 bp) that includes a G-box element (5′-CACGTG-3′; Figure 4a). Diploid yeasts containing the plasmids *B1.Wt-pTUY1H* and *AtbZIP44-pDEST®22* are able to grow in an auxotrophic medium lacking histidine, even in the presence of 15 mM 3-amino-1,2,4-triazole (3-AT), an inhibitor of the *HIS3* reporter gene, while growth of the negative control (*B1.Wt-pTUY1H* + *GFP-pDEST®22*) is blocked at 1 mM 3-AT (Figure 4b). To enquire whether the binding of the AtbZIP44 protein is through the G-box element (B1.Wt: 5′-CA**CGTG**-3′), a 2-bp mutation has been produced in such a G-box (B1.G-box mut: 5′-CA**a**G**g**G-3′). Diploid yeasts containing *AtbZIP44-pDEST®22* and the mutated *B1.G-box mut-pTUY1H* constructs are not able to grow in 3-AT concentrations higher than 1 mM, as occurs with the negative control with *GFP* (Figure 4b). All these results indicate that the G-box element of the conserved block B1 is specifically recognized by the AtbZIP44 transcription factor.

Another evidence of this specificity is that other bZIPs present in our library, AtbZIP11 (*At4 g34590*), its putative paralog with 78% similar amino acid residues, and AtbZIP4

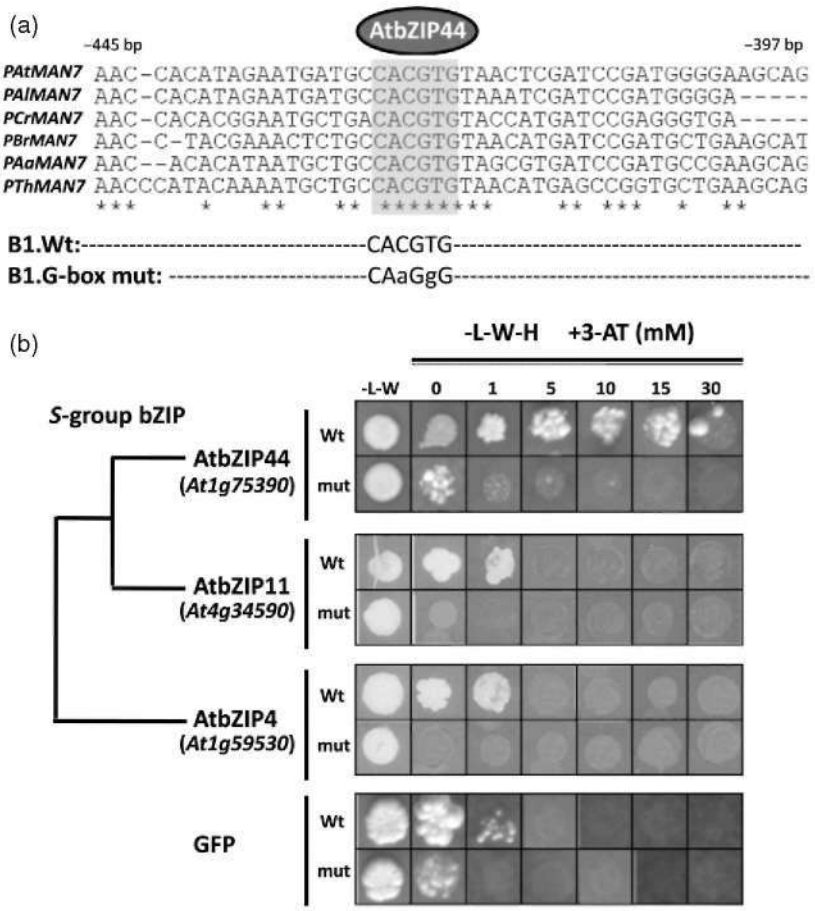

Figure 4. Yeast one-hybrid screening with *B1.Wt-pTUY1H*.
(a) Alignment of sequences spanning −445 to −397 bp within block B1 from different Brassicaceae species. A conserved G-box sequence is shaded and a mutated version with 2-bp changes in the G-box sequence (B1.G-box mut) is indicated.
(b) AtbZIP44 specifically binds to the G-box sequence of the block B1. Yeast strains containing either the block B1 element (Wt) or a 2-bp mutation in the G-box (mut), have mated to strains containing the AD-AtbZIP44, AD-AtBIP11, AtbZIP4 or AD-GFP (negative control) constructs. Diploid cells were grown on auxotrophic media with increasing concentrations of 3-amino-1,2,4-triazole (3-AT).

(*At1 g59530*), which although it belongs to the same *S*-group of bZIPs (Jakoby *et al.*, 2002) is more distantly related to AtbZIP44 (only with 21% of identical residues), are not able to bind to block B1 or to its mutated version (Figure 4b).

AtMAN7 and *AtbZIP44* genes are co-expressed in the ME upon seed germination in *A. thaliana*

To determine if AtbZIP44 could be a transcriptional regulator of the *AtMAN7* gene, transcripts of both genes should be expressed in the same cells at the same time. In order to explore this, we have started by analyzing by RT-quantitative (q)PCR the presence of both mRNAs upon seed germination (Figure 5). Accumulation of *AtMAN7* transcript reaches a peak at 12 h and remains constant until 24 h, decreasing sharply at 36 h, and being almost undetectable at 48 h. Accumulation of the *AtbZIP44* transcripts starts at 6 h and this expression remains fairly constant at (4–8) $\times$ 10^{-3}/18s-RNA until 36 h, diminishing thereafter. The expression of its closely related *AtbZIP11* gene is one order of magnitude lower than that of *AtbZIP44*.

Our previous data have demonstrated that *AtMAN7* gene expression is restricted to the ME and to the radicle tip in the Columbia accession (Col-0) of germinating *A. thaliana* seeds, this expression disappearing soon after radicle emergence (Iglesias-Fernández *et al.*, 2011a). To determine if the spatial expression of the *AtbZIP44* and/or *AtbZIP11* mRNAs co-localizes with that of the *AtMAN7* gene, FISH experiments have been performed in germinating seeds at 18 h (Figure 6). Samples have been hybridized to specific antisense probes derived from the 3′-non coding sequences of *AtMAN7*, *AtbZIP44* and *AtbZIP11* (Table S4). In longitudinal sections, a strong signal for the *AtMAN7* transcripts is detected in the ME (Figure 6a,d), while that of the *AtbZIP44 mRNA* is localized in the ME and in the radicle (Figure 6b,e). The similar localization of both transcripts in the ME is compatible with a possible transcriptional regulation of the *AtMAN7* gene by the AtbZIP44 TF in these

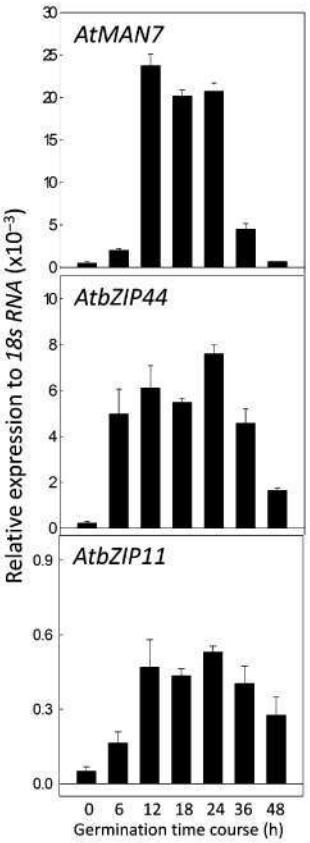

Figure 5. Expression analysis of *AtMAN7, AtbZIP44, AtbZIP11* in *Arabidopsis thaliana* germinating seeds. The RT-quantitative PCR data are normalized to the *18s-RNA* gene. Data are means ± standard error (SE) of three independent experiments.

cells. However, *AtbZIP11 mRNA* is faintly expressed at the radicle and it is not detected in any of the cells of the endosperm, thus excluding the possibility of the *AtMAN7* gene being regulated by AtbZIP11 at the ME (Figure 6c,f,i). As expected, no signal has been detected in seed sections hybridized with the corresponding sense probes (Figure 6g–i). Microscopic analysis of coarse sections (8 μm) of paraffin samples by differential interference contrast (DIC; Figure 6j–l) reveals that the cellular organization of the seed tissues has been adequately preserved. These data do not exclude other possible functions for AtbZIP44 in the radicle upon germination.

The transcription factor AtbZIP44 affects seed germination and expression of the *AtMAN7* gene

The oex lines oex-bZIP44-1 and oex-bZIP44-2 generated in the Col-0 accession have been used to explore not only the *AtMAN7* transcript accumulation but also their seed germination kinetics (Figure 7). Accumulation of *AtMAN7* transcript is two-fold and three-fold higher at 12 h in oex-bZIP44-1 and oex-bZIP44-2, respectively (Figure 7a). The germination assays performed with the homozygous oex-bZIP44.1-2 seeds appear in Figure 7(b–d), where two parameters have been scored: SCR and ER when the radicle protrudes. When establishing the germination kinetics of the two oex-bZIP44 lines, there are significant differences at t_{50} (time to get 50% of SCR or 50% of ER; see insets of Figure 7c,d) as compared with the t_{50} values for the wild type (Col-0).

Since KO mutant lines for *AtbZIP44* could not been found in the Columbia background, where most of the molecular and physiological *MAN* gene research has been carried out, other collections of *A. thaliana* accessions have been searched and two T-DNA insertion lines for *AtbZIP44* have been found in the Wassilewskija (Ws-4) background: Flag-295F02 (KO bZIP44-1) and Flag-270C06 (KO bZIP44-2), at positions +281 and +242 from the ATG translation initiation, respectively (Figure 8a). As a control, a KO mutant for *AtMAN7* in the Ws-4 background has also been analyzed (KO MAN7-1: Flag-430B06; Figure 8a). To explore if these T-DNA insertion mutants in *AtbZIP44* have an effect on the expression levels of *AtMAN7*, RT-qPCR analyses have been done in KO bZIP44-1 and KO bZIP44-2 homozygous seeds during germination (0, 36, 42, 60, 72 h; Figure 8b). Accumulation of *AtMAN7* transcript decreases to almost 30% in the bZIP44 mutants as compared with the wild type at 36 h, and remains at this level during the rest of the period analyzed (up to 72 h). As expected, the *AtMAN7* and *AtbZIP44* transcripts are barely detected in their corresponding KO lines and the KO mutant *AtMAN7* line is not affected in the expression of the *AtbZIP44* transcript (Figure 8b). Germination assays have been performed with the homozygous KO bZIP44.1-2 seeds and SCR and ER have been scored (Figure 8c,d). Germination kinetics of the two KO bZIP44 lines differ significantly at t_{50} values (see insets of Figure 8c,d) as compared with the wild-type (Ws-4) control and this is particularly remarkable when scoring for the t_{50} of the SCR.

DISCUSSION

In this study, we have established the phylogeny of the endo-β-mannanase genes among several Brassicaceae species and analyzed the conserved *cis*-elements in the promoters of the *MAN7* orthologous genes with the goal of establishing the *cis–trans* regulatory code of the *AtMAN7* gene; previous studies have demonstrated that the *AtMAN7* gene has an important role during germination. We have demonstrated here that the AtbZIP44 TF, but not its closely related AtbZIP11, affects seed germination and expression of the *AtMAN7* gene, most probably by specifically interacting with a G-box (5′-CACGTG-3′; −428 bp to −423 bp) within one of the evolutionarily conserved *cis*-elements (block B1) of the *AtMAN7* gene promoter.

Non-coding sequences of orthologous genes diverge rapidly during evolution, except for motifs that are important for function (Vavouri and Elgar, 2005). Through a

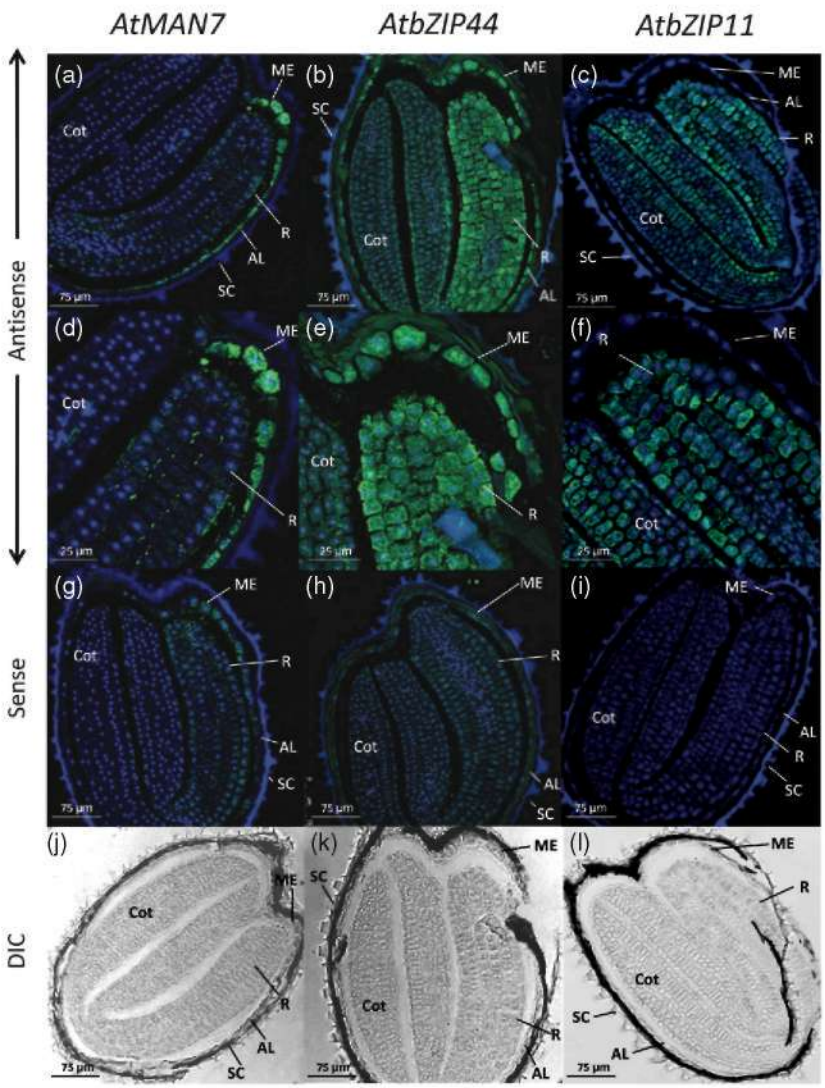

Figure 6. Fluorescence *in situ* mRNA hybridization (FISH) analysis of *AtMAN7* (a, d, g), *AtbZIP44* (b, e, h), and *AtbZIP11* (c,f, i) transcripts in germinating seeds (18 h). Longitudinal sections of germinating seeds (a–c, g–l). Close-up of the radicle tip and the micropylar zone (d–f). Control sense probes of germinating seeds (g–i). Merged picture of the anti-mouse IgG-Alexa Fluor 488 with 4′,6-diamidino-2-phenylindole staining to reveal nuclei (a–i). Differential interference contrast (DIC) images (j–l). AL, aleurone layer; Cot, cotyledon; ME, micropylar endosperm; SC, seed coat; R, radicle.

phylogenetic shadowing analysis of the promoters of *MAN7* orthologous genes from six Brassicaceae species, two highly conserved motifs in these promoter regions have been identified (blocks A and B; Figure 1). In germinating transgenic *A. thaliana* seeds expressing the *uidA* reporter under the control of the whole *AtMAN7* gene promoter (−844 bp; *PMAN7-1::uidA*), GUS activity is restricted to the ME at 24 h (Figure 2), which is in agreement with our previous mRNA *in situ* analysis of the *AtMAN7* gene (Iglesias-Fernández *et al.*, 2011a). Serial deletions of the *AtMAN7* promoter constructs reveal the functional relevance of the conserved block B. While absence of block A, such as occurs in the *PMAN7-2::uidA* construct (−509 bp) does not affect substantially the expression of the *uidA* reporter gene, when block B is eliminated no GUS can be detected (*PMAN7-3::uidA* lines; −280 bp; Figures 2c–e and S3). This indicates that block B contains crucial regulatory elements for the transcriptional regulation of the *AtMAN7* gene.

When the conserved block B1 has been used as bait in Y1H experiments against a TF library of about 1200 TFs (Castrillo *et al.*, 2011), the AtbZIP44 protein (*At1 g75390* gene) has been identified as interacting with this *cis*-element (Figure 4b). The specificity of the binding through the G-box within block B1 has been documented, because when we mutate the G-box (5′-CAaGgG-3′) AtbZIP44 does not recognize this *cis*-element in the Y1H experiments. Moreover, neither AtbZIP11 (*At4 g34590*), its putative paralog with 78% similar amino acid residues, nor AtbZIP4 (*At1 g59530*) more distantly related to AtbZIP44 (only with 21% of identical residues), are able to bind to the element (Figure 4b). Homodimerization and heterodimerization are common among bZIPs. In particular, heterodimerization between AtbZIP44 with bZIPs of the C-group (AtbZIP10, AtbZIP25) has been previously described both in the yeast two-hybrid (Y2H) and in Arabidopsis protoplasts (P2H) systems (Ehlert *et al.*, 2006). However, in Y2H experiments, we could not find homodimers of AtbZIP44 although we

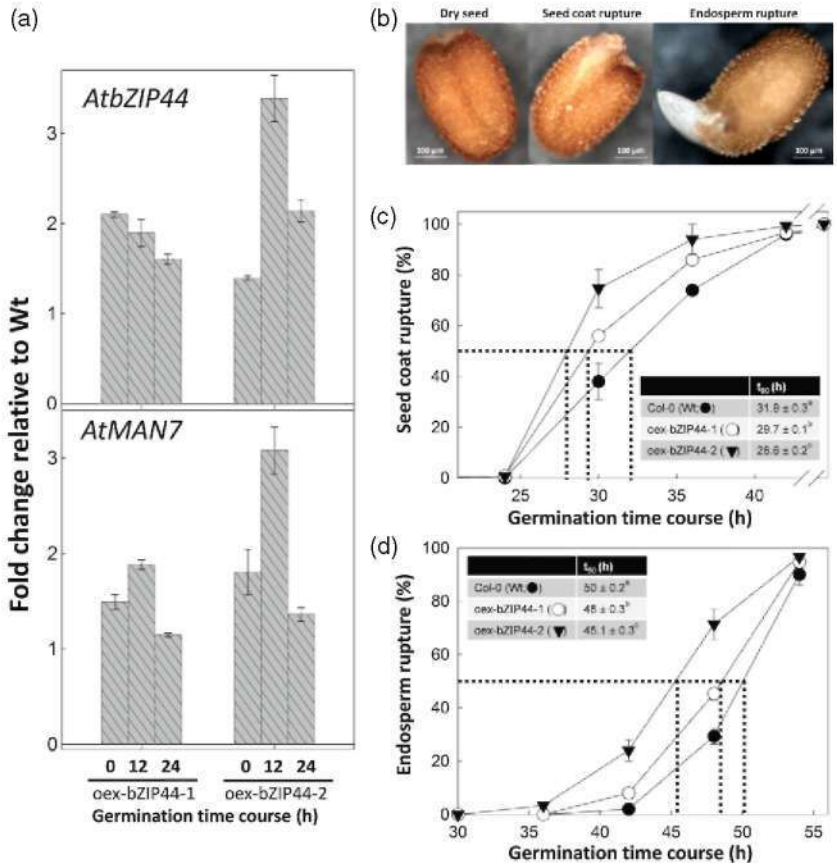

Figure 7. *AtMAN7* gene expression and germination kinetics in overexpression (oex) lines oex-bZIP44-1 and oex-bZIP44-2 in the Col-0 accession.
(a) Transcript analysis by RT-quantitative PCR of the *AtbZIP44* and *AtMAN7* genes upon seed germination normalized to the *18s-RNA* gene and refered to the wild-type (Wt) seed transcripts. Data are means ± standard error (SE) of three independent experiments.
(b) Different phases of *Arabidopsis thaliana* seed germination. Dry seed is the imbibed seed starting the testa rupture; germinated (*sensu stricto*) seed is when endosperm rupture occurs. (c) Seed coat rupture (SCR) time course of *A. thaliana* Wt (Col-0) and oex-bZIP44-1 and oex-bZIP44-2 germinating seeds. (d) Endosperm rupture (ER) time course of the same germinating seeds as in
(c). Wt, closed circles (●); oex-bZIP44-1, open circles (○); oex-bZIP44-2, closed triangles (▼). In the insets, the time necessary for 50% seed coat rupture or endosperm rupture (ER; t_{50}) is indicated. Data are means ± SE of three independent experiments. Significant differences between values are shown as different letters ($P < 0.05$).

could find heterodimers of AtbZIP44 with AtbZIP10 and AtbZIP25 (data not shown). AtbZIP10 and AtbZIP25 can form heterodimers between them, and are involved in regulation of seed storage protein genes upon silique development (Lara *et al.*, 2003); transcripts of *AtbZIP10* are hardly detected in germinating seeds and those of *AtbZIP25*, although present, seem not to be induced upon germination (https://www.genevestigator.com/gv/; Figure S4); Whether AtbZIP25 plays a role as co-regulator with AtbZIP44 of the *AtMAN7* gene, although improbable, will need further investigation.

As shown in Figure 8, the expression of *AtbZIP44* precedes radicle protrusion. Moreover, KO bZIP44 mutants have a slower germination than that of the corresponding wild type and the oex-bZIP44 lines display a faster germination, suggesting a role for the AtbZIP44 TF in the germination process and notably during SCR in the Ws-4 background. Besides, the mRNA *in situ* localization of the *AtbZIP44* and *AtMAN7* genes during seed germination is compatible with the transcriptional regulation of the *AtMAN7* gene by AtbZIP44; suppression of *AtbZIP44* expression, as occurs in the KO bZIP44.1-2 lines, not only lowers *AtMAN7* gene expression to 30% of the wild-type control, but these lines have a significantly slower germination. This indicates that AtbZIP44, although not the only TF involved, is not a minor transcriptional regulator of *AtMAN7*. A similar situation has been described in barley with BLZ2 that is a bZIP associated with a quantitative trait locus for yield that influences the accumulation of protein and carbohydrate in seeds (Oñate *et al.*, 1999; Haseneyer *et al.*, 2010). One possibility could be that AtbZIP44 was redundant with other bZIPs. AtbZIP11 could be a good candidate for that, because of its close phylogenetic relationship with AtbZIP44 (78% similar residues), but *AtbZIP11*

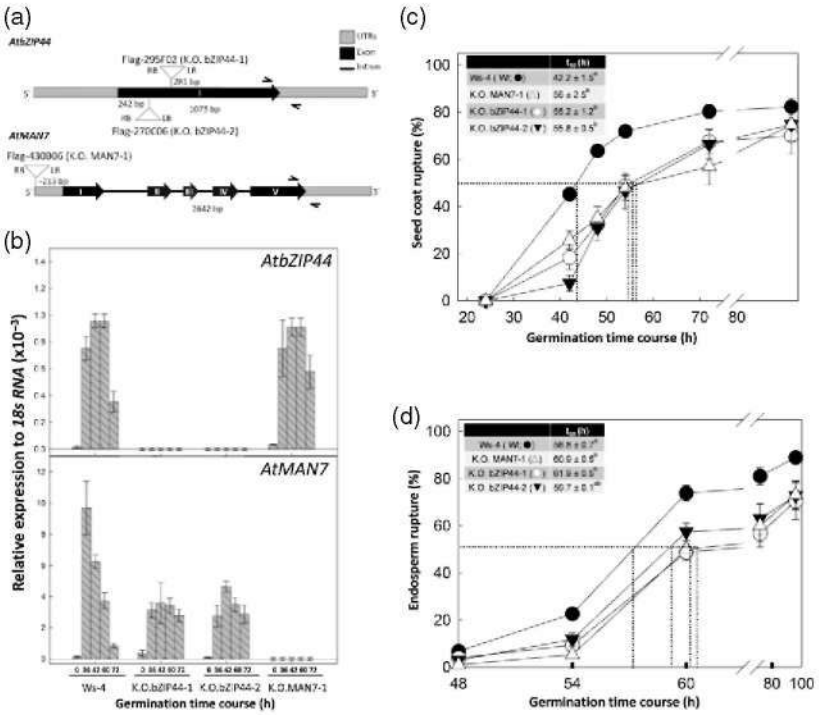

Figure 8. Knock-out (KO) mutant lines for *AtbZIP44* are negatively affected in *AtMAN7* gene expression and have altered their germination kinetics.
(a) Positions of the T-DNA insertions (indicated with a ▽) in the AtbZIP44 and AtMAN7 mutants analyzed. Horizontal arrows (⟷) represent the localization of the primers used for the RT-quantitative (q)PCR analysis.
(b) Transcript analysis by RT-qPCR of the *AtMAN7* and *AtbZIP44* genes expressed in wild type (Wt) and KO mutants at different time points of seed germination normalized to the *18s-RNA* gene. Data are means ± standard error (SE) of three independent experiments.
(c) Seed coat rupture time course of *Arabidopsis thaliana* Wt and T-DNA insertion mutant seeds. (d) Endosperm rupture time course of *A. thaliana* Wt and T-DNA insertion mutant seeds. Wt, closed circles (●); KO MAN7-1 (Flag-430B06), open triangles (△); KO bZIP44-1 (Flag-295F02), open circles (○); KO bZIP44-2 (Flag-270C06), closed triangles (▼). In the insets, the time necessary for 50% seed coat rupture or endosperm rupture (ER; t_{50}) is indicated. Data are means ± SE of three independent experiments. Significant differences between values are shown as different letters ($P < 0.05$).

transcript accumulation is quantitatively one order of magnitude lower than that of *AtbZIP44* and *AtbZIP11* is not localized in our FISH experiments to the ME where *AtMAN7* is expressed. Moreover AtbZIP11 does not bind to the G-box contained in block B1 of the *AtMAN7* gene promoter and neither bZIP do interacts in Y2H assays (our data not shown). Besides, *AtbZIP11* expression is not altered in the T-DNA insertion mutants KO bZIP44.1-2 (our unpublished results), thus discarding a cross-regulation between them. AtbZIP11 is known to regulate the expression of the *ASPARAGINE SYNTHETASE1* (*ASN1*) gene by the interaction with a specific G-box contained in the promoter of *ASN1*, and as occurs in our case with AtbZIP44 and *AtMAN7*, AtbZIP11 does not recognize the second G-box present in the *ASN1* promoter (Hanson *et al.*, 2008).

There are other bZIPs of the same *S*-group to which AtbZIP44 belongs, such as AtbZIP1 (*At5 g49450*), AtbZIP2 (*At2 g18160*), AtbZIP4 (*At1 g59530*) and AtbZIP53 (*At3 g62420*); this last one has been previously described to be a transcription activator of the albumin *2S2* gene during seed development (Alonso *et al.*, 2009). However, only the *AtbZIP2* gene is highly expressed during seed germination, as described in public arrays (Figure S4; https://www.genevestigator.com/gv/), but it does not interact with *AtbZIP44* (Ehlert *et al.*, 2006). Besides, none of these three TFs have been isolated in the Y1H screening using the B1-element as bait (our data not shown). Taken together, these data indicate that the paralogs of AtbZIP44, AtbZIP11 and AtbZIP2 are not implicated in the transcriptional regulation of *AtMAN7* expression upon seed germination.

Since the KO mutants (bZIP44-1 and bZIP44-2 in the Ws-4 background) have a slower germination and the oex-bZIP44 lines show a faster germination than their corresponding wild type, and the *AtbZIP44* expression appears in the ME as well as in the radicle, AtbZIP44 TF can influence not only the loosening of the CW in the ME upon germination, but could also be involved in elongation of the radicle cells. Furthermore, the greater difference between the delays of SCR in KO mutants compared with those of the ER suggests a stronger influence of AtbZIP44 in the SCR than in the ER. The SCR is related to the mechanical force exerted by the embryonic axis upon the seed coat when the GA content increases during seed imbibition. The mutant *ga1-3*, deficient in GA synthesis, has completely blocked both SCR and ER. On the contrary, the addition of ABA to the imbibition medium of wild-type

seeds entirely supresses ER but only delays SCR. It has been proposed that a low GA level provokes the over-accumulation of three DELLA repressor proteins (GAI, RGA and RGL2) that block testa rupture and eventually avoids ER (Müller *et al.*, 2006; Piskurewicz *et al.*, 2008). A positive GA/ABA balance is critical for germination to occur; GA synthesis starts shortly upon seed imbibition, which is essential for the rupture of both testa and endosperm tissues (Debeaujon and Koornneef, 2000; Weitbrecht *et al.*, 2011). Gibberellin promotes SCR through the stimulation of radicle growth and reduces the mechanical constraint conferred by the ME; both processes are influenced by the up-regulation of cell wall-modifying enzymes (Bewley, 1997; Leubner-Metzger, 2003). Phenotypic features of KO AtMAN7 lines upon seed imbibition, where both SCR and ER kinetics are slower than those of the wild type, point out for a role for *AtMAN7* in both testa rupture and in loosening of the CW endosperm that facilitates protrusion of the radicle during seed germination. Moreover, quantitative determination of GUS activity in germinating seeds of transgenic plants where the *uidA* reporter gene was under the control of several deletion constructs of the *AtMAN7* gene promoter shows a clear response of the *AtMAN7* promoter to GA while it did not respond to ABA (Figure 3). This GA stimulation occurs earlier in the germinating seeds of plants with the construct *PMAN7-2::uidA*, where block A has been eliminated, than in the *PMAN7-1::uidA* seeds; an indication that block B could be responsible for this GA response and that block A would contain some element interacting with a GA repressor. In *Solanum lycopersicum* seeds, induction of *LeMAN2* and *LeMAN1* genes by GA during germination and post-germination, respectively, has been reported (Gong and Bewley, 2008). Transcripts of the orthologous gene of *AtMAN7* in *Lepidium sativum* (*LesaMAN7*) are accumulated in the endosperm cap and to a lesser extent in the radicle during seed germination, and this induction is not inhibited by ABA (Morris *et al.*, 2011). In *A. thaliana* and *L. sativum*, endosperm weakening is mediated, at least in part, by GA-induced genes encoding cell wall remodeling proteins (Voegele *et al.*, 2011). It has been reported that *A. thaliana* transgenic seeds overexpressing the ortholog of *AtbZIP44* of soybean (*GmbZIP44*) presents a higher germination percentage than those of the wild type when imbibed with 1 μM ABA (Liao *et al.*, 2008). This result supports our view that AtbZIP44 positively affects seed germination, but, in our case, this effect seems to be independent of ABA. However, *Glycine max* belongs to the Leguminosae family whose seeds, lacking an endosperm, germinate in only one-step and ABA does not inhibit testa rupture but inhibits water uptake in the transition from germination to post-germination growth (Manz *et al.*, 2005). Thus, it could be possible that bZIP44 has a different role during germination of endospermic and non-endospermic seeds.

In this work, we have further demonstrated that the protein AtMAN7, with a predicted signal peptide in its deduced amino acid sequence, is exported to the peripheral regions of the cell (cell wall and plasma membrane; Figure S2). This result is in accordance with our previous proposed model for a possible cooperation between radicle and endosperm MAN enzymes in the dismantling of the ME CWs, thus facilitating radicle protrusion (Iglesias-Fernández *et al.*, 2011b). Recently, a large amount of heteromannan has been reported to be localized in the mucilage layer but not in the endosperm or in the radicle of germinating (3 h) *A. thaliana* seeds (Lee *et al.*, 2012). In this context, movement of the AtMAN7 protein through the endoplasmic reticulum to the periphery of the cell acquires a great relevance, since protein produced in the radicle tip and in the ME cells could be secreted to the apoplastic space, and thus be present in the mucilage layer, without discarding that MAN could also be important in softening the CWs at the ME cells.

In summary, the data presented here indicate that AtbZIP44 is a transcriptional regulator affecting seed germination '*sensu stricto*' and *AtMAN7* gene expression, and this positive regulation involves both loosening of the ME and rupture of the seed coat. The SCR is stimulated by the elongation of the radicle and by the secretion of CW hydrolytic enzymes, such as MAN, to the seed coat to provoke the hydrolysis of the mannans in the CWs. But the question of whether GA is the phytohormone behind the molecular control of *AtbZIP44* gene needs further investigation. Moreover, post-transcriptional regulation of AtbZIP44 needs to be considered, since all members of the bZIP *S1*-group, including AtbZIP44, contain an upstream open reading frame (uORF) that is involved in a post-transcriptional repression by sucrose (Weltmeier *et al.*, 2009). We can't rule out also the possibility that AtbZIP44 could be participating in the control of other genes in the radicle during seed germination, because their transcripts are also localized in the radicle besides being clearly expressed in the ME.

EXPERIMENTAL PROCEDURES

Plant material, growth conditions and germination assays

Seeds of the *A. thaliana* ecotypes Col-0 and Ws-4 and T-DNA insertion mutants in this latter ecotype obtained from the French National Institute for Agricultural Research (INRA), were identified from IJPB-INRA (http://www-ijpb.versailles.inra.fr) and they are: Flag-295F02 (KO bZIP44-1), Flag-270C06 (KO bZIP44-2), Flag-430B06 (KO MAN7-1). Homozygous plants for the T-DNA insertions were selected by PCR using a gene-specific primer and a primer derived from the left border (LB4) of the T-DNA (http://signal.salk.edu/tdnaprimers.2.html; Table S4).

Plant growth conditions and germination assays, in non-cold-stratified seeds, were performed as described (Iglesias-Fernández *et al.*, 2011a). Statistical analyses were done using the GERMINATOR package program (http://www.pph.wur.nl/UK/seedlab/resources/germinator; Joosen *et al.*, 2010).

Generation of transgenic lines

The promoter (−844 bp) of the *AtMAN7* gene was amplified from *A. thaliana* genomic DNA by nested PCR using oligonucleotide pairs containing *attB* sites (Table S4) for cloning into the pDONR®221 by the Gateway® BP recombination and then transferred by Gateway LR recombination (Invitrogen; http://www.invitrogen.com) into the destination vector pMDC163, containing the *uidA* reporter gene (Curtis and Grossniklaus, 2003). The same strategy was used to obtain the over-expression lines in the Columbia background. The *p35s::bZIP44* was cloned into the pMDC43, using the plasmid pDEST22® containing the bZIP44 ORF, as entry clone. All constructs were introduced into *Agrobacterium tumefaciens* strain C58C1 GV3101 by electroporation and these were used to transform *A. thaliana* (Col-0) by the floral dip method (Clough and Bent, 1998).

Histochemical GUS assays

Qualitative GUS staining assays were performed using the protocol described by Jefferson *et al.* (1987). Samples were cleared using Hoyer's light medium as described by Stangeland and Salehian (2002).

For quantitative GUS assays, seeds of two transgenic independent lines (representative of a total of 12 independent transgenic lines) were imbibed at different time periods in 3 ml of sterile water or with different hormonal treatments 50 μM GA_{4+7} and 1 μM ±ABA (Duchefa Biochemie, http://www.duchefa-biochemie.nl/).

In *Nicotiana benthamiana* leaves, suspensions of *A. tumefaciens* strain C58C1 GV3101 were infiltrated containing constructs described in Figure 2(a), the plasmid *pMDC32::35s::LUC* (Luciferase; Gateway technology) was used to normalize the data and *pBIN61::35s::P19* to avoid genome silencing (Voinnet *et al.*, 2003). Quantitative GUS assays were performed according to Barrero *et al.* (2009) and for quantification of luciferase activity a high-sensitivity Luciferase Reporter Gene Assay kit (Roche Diagnostics, http://www.roche.com/) was used, following the manufacturer's instructions. Fluorescence/luminescence were determined using Genios Pro 96/384 multifunction microplate reader (TECAN®, http://www.tecan.com/). For statistical analysis of quantitative GUS activity data, a Student's *t*-test was performed.

Subcellular localization

Translational fusion of *AtMAN7* to the GFP reporter was generated by cloning the ORFs into the *Bam*HI/*Xba*I restriction sites of a *psmRS-GFP* plasmid, that was obtained from a *pUC118* vector (U07649) in which the *35s-psmRS-GFP-Nos* was previously cloned into the *Hin*dIII–*Eco*RI sites (Davis and Vierstra, 1998). The generated constructs were: *P35s::AtMAN7-GFP* and *P35s::GFP* that was used as a control. Gold particles (1 μm), coated with the appropriate DNA constructs, were bombarded into freshly prepared onion epidermal peels with a biolistic helium gun device (DuPont PDS-1000; Bio-Rad Laboratories, http://www.bio-rad.com/). The expression of the fluorescent proteins in onion epidermal cells was observed after 24 h of incubation at 22°C in the dark (Barrero-Sicilia *et al.*, 2011). Images were captured with a Zeiss Axiophot fluorescence microscope (Leica, http://www.leica.com/).

Bioinformatic tools: dendrogram and phylogenetic shadowing

All the sequences, from five different species within the Brassicaceae family, used in this work, were obtained from the Phytozome v8.0 Database (Goodstein *et al.*, 2012; http://www.phytozome.net), except for those of the *Arabis alpina* genome that were produced by the TRANSNET KBBE consortium (Prof. P. Carbonero, Universidad Politécnica de Madrid, Madrid, Spain, personal communication). Major characteristics of the predicted endo-β-mannanase proteins are listed in Table S1 and promoter sequences of *MAN7* orthologous genes are found in Table S3.

The complete deduced amino acid sequences of the 37 *MAN* genes from the six Brassicaceae species were used to construct a phylogenetic dendrogram; signal peptide, molecular weight and isoelectric point predictions were done as described by Iglesias-Fernández *et al.* (2011a).

The Brassicaceae *MAN7* promoter sequences were analyzed by the mVista Shuffle-LAGAN program that was used to create pairwise alignments of these promoters (Frazer *et al.*, 2004; http://genome.lbl.gov/vista). Sequences of conserved regions within promoters were analyzed with T-coffee (Notredame *et al.*, 2000; http://www.ebi.ac.uk/Tools/msa/tcoffee). Plant *cis*-acting regulatory DNA elements were searched through the following databases: MotifFinder (http://www.genome.jp/tools/motif) and PlantCare (Lescot *et al.*, 2002; http://bioinformatics.psb.ugent.be/webtools/plantcare/html).

Yeast one-hybrid assays

To amplify by PCR the conserved block *B1* within the *AtMAN7* promoter and its mutated version (*B1.G-box mut*) we used the primers *AS-PAtMAN7.B1-HYB*/*S-PAtMAN7.B1-HYB* and *AS-PAtMAN7.B1-HYB*/*S-PAtMAN7.B1mut-HYB*, respectively (Table S4). Yeast one-hybrid screenings were performed as described by Castrillo *et al.* (2011).

Real-time quantitative PCR assays

Total RNA was purified from *A. thaliana* seeds at different time points of germination (up to 72 h) as described by Oñate-Sanchez and Vicente-Carbajosa (2008). The cDNA was synthesized from 1 μg of total RNA using the First-Strand Synthesis kit for RT-PCR (Roche Diagnostics) following the manufacturer's instructions. Samples were stored at −20°C until use.

The specific primers used in the RT-qPCR analysis appear in Table S5. We used *18s-RNA* to normalize the data (ΔC_T; Figures S5–S7). The RT-qPCR was performed in an Eco® Real-Time PCR System (Illumina, http://www.illumina.com/). For each 10-μl reaction, a 1 μl cDNA sample was mixed with 5 μl of FastStart Universal SYBR Green Master (ROX; Roche Diagnostics), 0.25 μl of each primer (final concentration 500 nM), plus sterile water up to the final volume. Samples were subjected to thermal-cycling conditions of 95°C for 10 min, 40 cycles of (10 sec at 95°C, 30 sec at 60°C of annealing and extension). The melting curve was designed to increase from 55 to 95°C; melting temperatures for each amplicon and primer efficiencies, estimated via a calibration dilution curve and slope calculation, are shown in Table S5. This analysis was performed with three different biological samples for each time-point and each one was duplicated. Expression levels were determined as the number of cycles needed for the amplification to reach a threshold fixed in the exponential phase of the PCR reaction (C_T; Pfaffl, 2001).

Messenger RNA FISH experiments

The protocol followed here was described by Iglesias-Fernández *et al.* (2011a) and by Testillano and Risueño (2009). Fragments of DNA (200–300 pb) derived from the 3'-non coding regions of the Arabidopsis genes analyzed have been used as probes (Table S4).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Phylogenetic tree and schematic distribution of conserved motives among the deduced protein sequences of the endo-β-mannanase proteins of the Brassicaceae species used in this study.

Figure S2. Subcellular localization of the AtMAN7 protein.

Figure S3. Histochemical localization of GUS in 24-h transgenic adult roots of *PMAN7-1::GUS, PMAN7-2::GUS, PMAN7-3::GUS.*

Figure S4. Heatmap representing expression levels of *AtbZIP44* (*At1 g75390*), *AtbZIP11* (*At4 g34590*) *AtbZIP1* (*At5 g49450*), *AtbZIP2* (*At2 g18160*), *AtbZIP4* (*At1 g59530*) *AtbZIP53* (At3 g62420), *AtbZIP10* (*At4 g02640*) and *AtbZIP25* (*At3 g54620*) during seed germination.

Figure S5. Transcription levels of housekeeping gene (*18s-RNA*), presented as Ct mean values, during seed germination of *Arabidopsis thaliana* Col-0 seeds and during silique development of *A. thaliana* Col-0.

Figure S6. Transcription levels of housekeeping gene (*18s-RNA*), presented as Ct mean values, during seed germination of *Arabidopsis thaliana* Ws-4, knock-out (KO) bZIP44-1, KO bZIP44-2 and KO MAN7-1 seeds.

Figure S7. Transcription levels of housekeeping gene (*18s-RNA*), presented as Ct mean values, during seed germination of *A. thaliana* Col-0, oex-bZIP44-1, and oex-bZIP44-2 seeds.

Table S1. Major characteristics of Brassicaceae predicted endo-β-mannanase proteins.

Table S2. Sequences of conserved amino acids motives (MEME; Bailey *et al.*, 2006) of the endo-β-mannanases from the Brassicaceae species in Figure S1.

Table S3. Promoter sequences of *MAN7* genes of the Brassicaceae used in the phylogenetic shadowing analysis.

Table S4. List of primers used for cloning, for probe synthesis in fluorescent *in situ* hybridization and for genotype T-DNA insertion mutant lines.

Table S5. Characteristics and sequences of the primers used in the RT-qPCR analysis.

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